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PURIFICATION AND CHARACTERIZATION OF A CARBOXYL ESTER HYDROLASE FROM HUMAN PANCREATIC JUICE

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Summary

A carboxyl ester hydrolase has been purified 20-fold from human pancreatic juice. It is a glycoprotein with a molecular weight of 100 000. It contains 9% neutral and amino carbohydrates and the amino acid composition is characterized by a high content of proline residue (12.7%).

The enzyme catalyzes the hydrolysis of *p*-nitrophenylacetate and the activity increases in the presence of biliary salts; V is not modified but K_m is decreased 10 times by addition of biliary salts. The enzyme migrates on Sephadex G-200 as a protein with a molecular weight of 300 000. This behaviour does not seem to be due to a polymerization but to a peculiar configuration of the enzyme.

Introduction

The presence in pancreas of an esterase activity different from lipase activity was reported many years ago. In 1958, Sarda and Desnuelle separated the two activities by electrophoresis of porcine pancreatic extracts and demonstrated that esterase hydrolyzes soluble methylbutyrate while lipase attacks the same substrate only when emulsified, above saturation [1]. In 1968, Morgan et al. [2] characterized and separated by gel filtration those two activities from rat pancreatic juice. Recently the enzyme hydrolyzing water-soluble esters and called carboxyl ester hydrolase (carboxylic-ester hydrolase, EC 3.1.1.1) has been purified by Erlanson [3]. This protein, with a molecular weight of 70 000, has a wide substrate specificity and hydrolyzes water-soluble esters like *p*-nitrophenylacetate [4] as well as water-insoluble esters when dispersed with bile salts. A similar enzyme activity has been demonstrated in human pancreatic juice and intestinal contents [5,6]. This activity was separated from

Abbreviation: MES, 2-(*N*-morpholino)ethane sulfonic acid.

lipase by gel filtration on Sephadex G-100 and was shown to parallel a cholesterol esterase activity, though the enzyme responsible for this activity has not been isolated.

In this paper, we describe the purification of a carboxyl ester hydrolase characterized by its activity on *p*-nitrophenylacetate and isolated from human pancreatic juice. We also report some molecular and catalytic properties of this enzyme and discuss the amino acid composition. This had never been determined for a pancreatic carboxyl ester hydrolase.

Material and Methods

Materials. Pure human pancreatic juice was collected by catheterization of the pancreatic duct as previously described [7]. Only samples devoid of free proteolytic activity were used.

Antiserum to human pancreatic juice was prepared by immunization of rabbits with a mixture of pancreatic juices according to the method of Henry et al. [8].

p-Nitrophenylacetate was obtained from Sigma, sodium taurocholate and taurodeoxycholate from Calbiochem. Agar was purchased from Difco, Brij 35 from Pierce and Sephadex from Pharmacia Fine Chemicals.

Protein determination. Protein concentration was determined spectrophotometrically at 280 nm using during the different steps of purification, a mean extinction coefficient $E^{1\%}_{1cm}$ equal to 20.0. On the purified enzyme, the extinction coefficient was 14.5, value determined experimentally with the protein concentration given by the amino acid analysis.

Enzyme assays. The hydrolysis of *p*-nitrophenylacetate was measured spectrophotometrically at 400 nm using 0.33 mM as substrate concentration according to the method described by Erlanson [4]. The used extinction coefficient of *p*-nitrophenol was 16 300 $M^{-1} \cdot cm^{-1}$ [9]. Specific activities are given by the number of μmol of *p*-nitrophenylacetate hydrolyzed per min per mg of protein. Lipase activity was measured on an emulsion of olive oil by potentiometric method [10].

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis were performed in 7.5% polyacrylamide gels in the presence of sodium dodecyl sulfate according to the Weber and Osborn technique [11]. The reference proteins used for the molecular weight determination were: porcine aminopeptidase (mol. wts. of the 3 subunits: 130 000, 97 000, 49 000) kindly provided by S. Maroux, serum albumin (67 000) catalase (60 000) ovalbumin (45 000) and aldolase (37 000) purchased from Serva.

Filtration on Sephadex G-200. A calibrated column (0.9 × 200 cm) was used for the determination of the molecular weight by filtration on Sephadex G-200 according to the method of Andrews [12]. Filtration was performed in a 10 mM Tris-HCl/500 mM NaCl buffer (pH 7.8).

The reference proteins used were: liver carboxyl ester hydrolase (molecular weight 163 000) from Sigma, human lipase (49 000) kindly provided by A. De Caro, soybean trypsin inhibitor (21 000) and cytochrome *c* (12 400) from Worthington. The void volume was determined by filtration of DNA from *Escherichia coli* (Sigma).

Immunoelectrophoresis. Immunoelectrophoresis was performed on agar plates (1.5% in agarose) in a 0.05 M veronal acetate buffer (pH 8.6). Antiserum to human pancreatic juice was used for the precipitin reaction.

Ultracentrifugation assays. Assays were conducted by equilibrium centrifugation at 20°C in a Spinco-Beckman analytical ultracentrifuge Model E equipped with a scanner, according to the method of Yphantis [13].

Amino acid composition. Duplicate samples containing 2.5 nmol protein were hydrolyzed in evacuated sealed tubes with 1 ml of 6 M HCl at 100°C for 24, 48 and 72 h. Amino acid analyses were performed using a Jeol (JLC-5AH) amino acid analyzer. Half-cystine was determined as carboxymethyl-cysteine after reduction and carboxymethylation of the protein [14] and tryptophan by the method of Matsubara and Sasaki [15]. Calculated serine and threonine are extrapolations to zero time of hydrolysis. The integral values were calculated on the basis of the molecular weight of 100 573 derived from the amino acid composition according to the method developped by Delaage [16].

NH_2 residue determination. The amino terminal residue was determined by the dansyl technique of Gray [17]. Dansyl-amino acids were identified by polyamide thin-layer chromatography [18].

Carbohydrate determination. The presence of carbohydrates was investigated by the periodic acid-fuschin staining on polyacrylamide gels after electrophoresis [19]. Neutral sugars were estimated by the phenol/sulfuric acid method [20]. Identification and quantitation of amino sugars were performed on a Jeol amino acid analyzer (JLC-5AH) after 4 h hydrolysis with 4 M HCl.

Results

Purification procedure. Human pancreatic juice proteins were submitted to a gel filtration on Sephadex G-100 (Fig. 1) as previously described for the isolation of human trypsinogens [21]. Benzamidine was added to the column buffer to prevent any trypsinogen activation. Carboxyl ester hydrolase was eluted with the high molecular weight proteins ahead of lipase activity. Fractions devoid of lipase activity were pooled (pool A) and proteins were precipitated by addition of solid $(NH_4)_2SO_4$ to 80% saturation. The precipitate was dissolved in distilled water and equilibrated in a 10 mM Mes buffer (pH 6.5) 100 mM NaCl by gel filtration on a Sephadex G-25 column. The proteins were then applied to a (0.9 × 30 cm) column of CM-sephadex equilibrated in the same buffer (Fig. 2). After the elution of non-adsorbed proteins, carboxyl ester hydrolase was eluted by a linear concentration gradient of NaCl (100—300 mM) as a single peak with a constant specific activity of 40. The purification steps are summarized in Table I.

Criteria of homogeneity. The purified carboxyl ester hydrolase displayed a single band by 0.1% SDS polyacrylamide gel electrophoresis.

It gave a single precipitin line by immunoelectrophoresis against an antiserum to the total proteins of human pancreatic juice. Moreover, no sign of heterogeneity could be detected by ultracentrifugation.

Molecular weight determination. 0.1% SDS polyacrylamide gel electrophoresis gave a molecular weight of 100 000 ± 1000. With or without treatment of the protein with β-mercaptoethanol, the results were the same, which shows

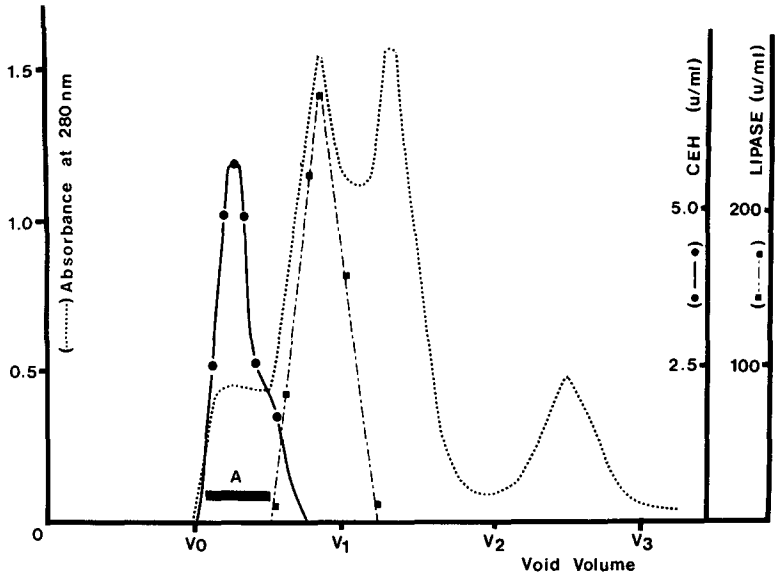

Fig. 1. Filtration of human pancreatic juice on Sephadex G-100. The column (2 X 120 cm) was equilibrated and developed with 500 mM NaCl 1 mM benzamidine 10 mM Tris-HCl (pH 8.0). The sample contained 120 mg proteins in 15 ml buffer. Void volume: 120 ml. CEH, carboxyl ester hydrolase.

that human carboxyl ester hydrolase contains a single polypeptide chain. The molecular weight determination by equilibrium centrifugation by the method of Yphantis gave the value of 98 100 ± 1000. The values calculated on the basis of the amino acid analysis were 100 660 — 100 823 (Table II).

The molecular weight of carboxyl ester hydrolase was also estimated by gel filtration on Sephadex G-200 in a high ionic strength buffer (10 mM Tris-HCl/

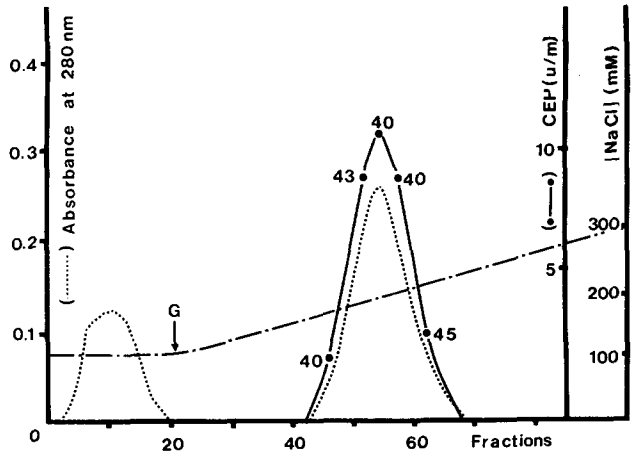

Fig. 2. Chromatography of carboxyl ester hydrolase on CM-Sephadex. The column (0.9 X 30 cm) was equilibrated with 100 mM NaCl 10 mM Mes buffer (pH 6.5). At the fraction indicated by G, a linear concentration gradient (100—300 mM NaCl) was applied to the column. (gradient chamber: 110 ml; 1.7 ml fractions; sample: 5 mg protein of pool A). Numbers on the unit profile indicate specific activities of carboxyl ester hydrolase. CEH, carboxyl ester hydrolase.

TABLE I

PURIFICATION OF HUMAN CARBOXYL ESTER HYDROLASE FROM PANCREATIC JUICE

Units are measured on *p*-nitrophenylacetate and expressed in μmol hydrolyzed per min.

Purification steps	Protein (mg)	Units	Yield (%)	Specific activity
Pancreatic juice	360	720	100	2
Sephadex G-100	23.6	600	83	26
CM-Sephadex	9.5	380	62	40

500 mM NaCl, pH 8.0). The observed k_{av} was 0.13 corresponding to a mol. wt. of 300 000. This value was previously reported to be the molecular weight of human carboxyl ester hydrolase [5]. All other determinations of molecular weight have led to the value of 100 000 and, thus, it is probable that the enzyme behaves abnormally on Sephadex. This behaviour cannot be explained by polymerization of the enzyme, since the same elution pattern is obtained by filtration in a low ionic strength buffer (10 mM Tris-HCl, pH 8.0) or in the presence of a detergent (1% Brij 35). This abnormal behaviour on carboxyl ester hydrolase could be due to a peculiar structure of the protein.

Amino acid composition, extinction coefficient and N-terminal residue. The results of the amino acid composition of carboxyl ester hydrolase are summarized in Table II. The protein is characterized by a low content of half-cystine (7), histidine (12) and methionine (16) and a very high content of proline (121) which represents 12.7% of the total number of amino acids.

The extinction coefficient of the protein was found to be 14.5.

The N-terminal residue of the polypeptide chain is alanine.

Determination of carbohydrate content. A positive periodic acid-fuschin

TABLE II

AMINO ACID COMPOSITION OF HUMAN CARBOXYL ESTER HYDROLASE

Amino acid	Residues/mol
Lysine	47.8
Histidine	12.0
Arginine	25.1
Aspartic acid	100.8
Threonine	75.9
Serine	53.1
Glutamic acid	59.6
Proline	120.8
Glycine	94.3
Alanine	88.6
Half-cystine	6.8
Valine	67.8
Methionine	16.3
Isoleucine	34.7
Leucine	57.6
Tyrosine	35.5
Phenylalanine	34.3
Tryptophan	17.1
Total	949—950
Mol wt.	100 660—100 823

reaction on polyacrylamide gel has demonstrated that human carboxyl ester hydrolase is a glycoprotein. Neutral carbohydrates have been evaluated to 5.2% in weight. The protein contains also amino sugars: 4.3 mol glucosamine and 17 mol of galactosamine per mol protein which represents 3.8% in weight. The presence of sialic acid has not been investigated.

Enzyme stability. Purified carboxyl ester hydrolase looses 50% of activity upon lyophilisation. The enzyme is stored at −20°C in pellets obtained by precipitation of proteins with ammonium sulfate at 0.8 saturation.

Kinetic parameters of p-nitrophenylacetate hydrolysis: effect of biliary salts. The kinetic parameters of *p*-nitrophenylacetate hydrolysis by carboxyl ester hydrolase were determined in the conditions described in Material and Methods, in the presence of 6 mM sodium taurocholate. From a Lineweaver-Burk plot K_m was found equal to 0.44 mM and V to $7 \cdot 10^{-2}$ μmol $\cdot$ s^{-1}. The calculated catalytic constant k_{cat} was $2.1 \cdot 10^{-2}$ s^{-1}. The same experiment performed in the absence of sodium taurocholate showed a modification of K_m which increased 10-fold to reach 4.5 mM while V was unaffected. The presence of biliary salts increases the affinity of carboxyl ester hydrolase for *p*-nitrophenylacetate. Fig. 3 shows that the activator effect of biliary salt starts with a very low concentration, close to 10^{-3} mM and that the effect is the same in the presence of sodium taurocholate or sodium taurodeoxycholate. The maximal activity (slightly higher in the presence of sodium taurocholate) is obtained for a concentration of 0.3 mM and for higher concentration of biliary salts the activity is stable and independent of the critical micellar concentration.

pH optimum. The pH profile of purified carboxyl ester hydrolase was mea-

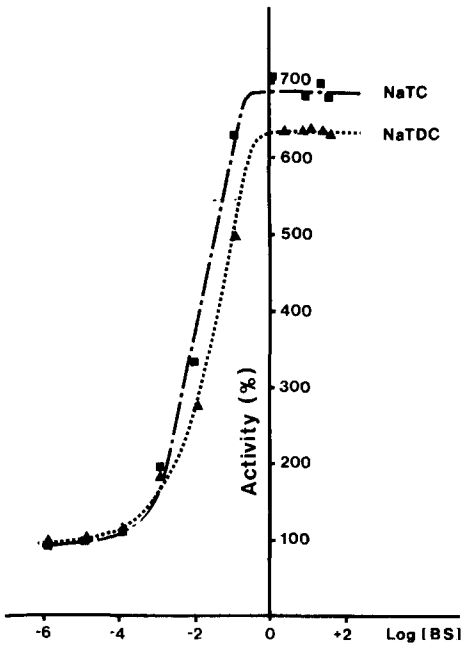

Fig. 3. Carboxyl ester hydrolase activity on *p*-nitrophenylacetate in relation to biliary salts concentration. The experiments were performed in the presence of sodium taurocholate (■— · — ·■) and sodium taurodeoxycholate (▲- - - - -▲). Concentration of biliary salts, [BS], was given in mM.

sured using *p*-nitrophenylacetate as substrate and has shown a maximal activity at pH 8.0.

Effect of salt concentration on esterase activity. The addition of NaCl or KCl in the assay increases the activity of carboxyl ester hydrolase on *p*-nitrophenylacetate until a plateau level of maximal activity corresponding to twice the activity of the enzyme in the absence of salts. This maximal activity is obtained with 0.25 M NaCl or KCl.

Inhibition by diisopropylfluorophosphate. The purified carboxyl ester hydrolase was very sensitive to diisopropylfluorophosphate and 100% inhibited at 10^{-4} M for 1 h.

Discussion

Human carboxyl ester hydrolase has been purified from pancreatic juice by a simple chromatographic procedure including two steps: a filtration on Sephadex G-100 at pH 8.0 and a chromatography on CM-Sephadex at pH 6.5. The enzyme represents 4% of the proteins of human pancreatic juice. It is a glycoprotein with a relatively high carbohydrate content (9%). The molecular weight determined by SDS polyacrylamide gel electrophoresis and ultracentrifugation or calculated on the basis of the amino acid composition was 100 000. This value is higher than that of the rat enzyme (70 000) and differs markedly from the molecular weight of 300 000 previously reported by Erlanson and Borgstrom [6], calculated from gel filtration of human pancreatic juice. Our experiments have demonstrated that human carboxyl ester hydrolase has an abnormal behaviour during filtration on Sephadex G-200 and that molecular weight cannot be determined by this procedure. The amino acid composition has pointed out a particularly high content of proline residues (12.7% of total amino acids). This result suggests that the protein has a limited helicoidal structure. This structure could be partially responsible for the abnormal behaviour of the protein on Sephadex.

The inhibition of the enzyme by diisopropylfluorophosphate indicates that human carboxyl ester hydrolase is a serine esterase like most of the known carboxyl esterases [22] and different from lipase whose an essential serine involved in catalysis is now controversial [23].

Like its homologous enzyme in rat, human carboxyl ester hydrolase hydrolyzes *p*-nitrophenylacetate, and bile salts strongly stimulate its esterase activity. When normalized to standard conditions the specific activities of the rat and human enzymes are respectively 81 and 40. But in our optimal conditions of assay for the human enzyme (0.5 M Tris-HCl/0.25 M NaCl, pH 8.0) the specific activity reached 94, which makes the activities of the two enzymes very close. However, the effects of biliary salts are different. Sodium taurocholate or taurodeoxycholate (in concentration well below the critical micellar concentration) increases 10-fold the affinity of human carboxyl ester hydrolase for *p*-nitrophenylacetate, whereas the rat enzyme requires for its maximal activity biliary salts in concentration above the critical micellar concentration and variable with the nature of bile salt [3]. In the presence of 6 mM sodium taurocholate the affinity of human esterase for *p*-nitrophenylacetate is 10 times that of the rat esterase [4]. However the relatively high values of each K_m sug-

gest that *p*-nitrophenylacetate is a poor substrate for both enzymes and probably very different from the physiological substrate. Studies on the specificity of human carboxyl ester hydrolase are in progress to investigate the biological activity of this pancreatic enzyme.

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